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CAUSAL AGENT FOR DISEASE SYMPTOMS ON PASSIONFLOWER IN ADIM, CROSS RIVER STATE, NIGERIA

E.E. EKPIKEN, O.I. EYONG and A.O. ADEDIJI[1]

Department of Plant Science and Biotechnology, University of Cross River State, Calabar, Cross River State, Nigeria
[1]Research Office, Pan African University Life and Earth Sciences Institute (including Health and Agriculture), Ibadan, Oyo State, Nigeria
Corresponding author: emmanuelekpiken09@gmail.com

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ABSTRACT

The stinking passionflower (*Passiflora foetida* L.) is generally regarded as a weed, although its tender fruits are consumed by a few; while the plant is mostly regarded for its medicinal properties. Cucumber mosaic virus is known not just to cause significant losses among crops, but also present in many weeds species. The objective of this study was to identify the causal agent of the disease symptoms observed from *P. foetida* in Adim, Cross River State, Nigeria. Leaves of *P. foetida* with virus symptoms were collected from various locations in Adim. Antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) and reverse transcription polymerase chain reactions (RT-PCR) were used for virus detection and characterisation. The symptomatic leaves reacted positively with the CMV antisera in ACP-ELISA and sequences from RT-PCR products showed the highest identity (98.5%) with a CMV isolate from *Xanthosoma* sp. (MG021460) in Uganda. Phylogenetic reconstruction aggregated the isolates with other Nigerian CMV sequences in a separate uncharacterised subgroup, closest to MH798809 (Nigeria), MW655577 (Nigeria) and HQ874434 (Austria). The tree also revealed the isolates as members of subgroup IB. This study provides the first molecular evidence of CMV infection on *P. foetida* in Nigeria and adds to the list of possible natural host for the virus in the country.

Key Words: ACP-ELISA, Cucumber mosaic virus, *Passiflora foetida,* RT-PCR, Subgroup IB

RÉSUMÉ

La passiflore puante (Passiflora foetida L.) est généralement considérée comme une mauvaise herbe, bien que ses fruits tendres soient consommés par quelques-uns; tandis que la plante est surtout considérée pour ses propriétés médicinales. Le virus de la mosaïque du concombre est connu non seulement pour causer des pertes importantes parmi les cultures, mais également pour de nombreuses espèces de mauvaises herbes. L'objectif de cette étude était d'identifier l'agent causal des symptômes de la maladie observés chez *P. foetida* à Adim, dans l'État de Cross River, au Nigéria. Les feuilles de *P.*

foetida présentant des symptômes viraux ont été collectées dans divers endroits d'Adim. Un test immuno-enzymatique sur plaque recouverte d'antigène (ACP-ELISA) et des réactions en chaîne par polymérase par transcription inverse (RT-PCR) ont été utilisés pour la détection et la caractérisation du virus. Les feuilles symptomatiques ont réagi positivement avec les antisérums CMV en ACP-ELISA et les séquences des produits RT-PCR ont montré la plus forte identité (98,5 %) avec un isolat CMV de Xanthosoma sp. (MG021460) en Ouganda. La reconstruction phylogénétique a regroupé les isolats avec d'autres séquences nigérianes de CMV dans un sous-groupe distinct non caractérisé, le plus proche de MH798809 (Nigéria), MW655577 (Nigéria) et HQ874434 (Autriche). L'arbre a également révélé que les isolats étaient membres du sous-groupe IB. Cette étude fournit la première preuve moléculaire d'une infection par le CMV sur *P. foetida* au Nigeria et s'ajoute à la liste des hôtes naturels possibles du virus dans le pays.

Mots Clés: ACP-ELISA, virus de la mosaïque du concombre, *Passiflora foetida*, RT-PCR, sous-groupe IB

INTRODUCTION

Wild water lemon or stinking passionflower (*Passiflora foetida* L.) belongs to the family Passifloraceae. It is an herbaceous climber weed, commonly found in the southern part of Nigeria, particularly in Adim, Cross River State, where it forms a dense ground cover which prevent growth, development and delayed establishment of other species, especially in rice and cotton fields; grain legumes and fallows (Takim *et al*., 2012).

The plant can also be found in waste and disturbed sites, cultivated rice fields, plantations and gardens. Due to its ubiquitous nature, it is sometimes described as an environmental weed. Although its fruits are technically edible, they are generally considered unpalatable due to their strong and unpleasant odour. They are uncommonly consumed due to a thin pulp.

Kumar *et al*. (2016) asserted that the plant is used to treat vomiting, eczema and chronic ulcers, while its fruits are used as blood purifier and leaves are used to cure asthma and hysteria. Root decoction are used in hysteria and other mental disorders; while leaves ash are mixed with vegetable oil to make paste, which is applied externally to cure scabies and on the head to cure giddiness and headache. It has been reported that all parts of *P. foetida* such as leaf, root, stem and fruit have tremendous medicinal properties, making the plant a hot spot for new drug discovery in the pharmaceutical industry (CABI, 2013). Pandith *et al.* (2022) reported that traditionally, healers use the plant to address conditions such as asthma, skin diseases, digestive issues and headaches.

In a proximate composition analysis, Odewo *et al.* (2014) revealed the plant to contain crude protein (25.83 to 26.05%), crude fibre (9.55 to 90.61%), crude fat (2.87 to 2.98%), ash (28.55 to 28.84%), carbohydrate (40.46 to 40.69%) and moisture (1.79 to 1.96%).

There is limited information available related to viruses that specifically infect *P. foetida*. However, Passiflora species are known to be susceptible to a range of viruses that can cause significant damage to the plants. Some of these viruses include Passiflora virus Y described from *P. foetida* (Parry *et al*., 2004). Passiflora latent carlavirus was also reported in Australia in wild Passiflora species (Pares *et al*., 1997). A begomovirus, Melochia yellow mosaic virus was reported to infect Passiflora plants in Brazil (Spadotti *et al*., 2019). Colariccio *et al*. (2020) reported a potyvirus, Cowpea aphid borne mosaic virus to infecting *P. edulis* in Brazil.

Other viruses described to have infected the plant genus include *Tymovirus,* Passion fruit yellow mosaic virus (Crestani *et al*., 1986); Passion fruit woodiness virus (Arogundade *et*

al., 2018); Passion fruit leaf mosaic virus and Passion fruit severe leaf distortion virus (Fischer and Resende, 2008; Colariccio *et al*., 2018). A potyvirus associated with passion fruit severe mosaic and fruit woodiness disease in Thailand, was identified as a strain of Telosma mosaic virus (Chiemsombat *et al*., 2014).

Another virus that has been found to infect members of Passiflora species is Cucumber mosaic virus (CMV) (Arogundade *et al*., 2023): which is a member of the Bromoviridae family and is transmitted by several species of aphids in a non-persistent manner (Palukaitis and Garcia-Arenal, 2003). The virus elicits mosaic symptoms on the leaves, stunted growth, and reduced fruit production. The objective of this study was to identify the causal agent of the symptoms observed on *P. foetida* leaves in Adim, Cross River State in Nigeria.

MATERIALS AND METHODS

Virus isolates and extraction of total RNA. A total of twenty young leaves of *P. foetida,* showing characteristic mosaic, leaf malformation and stunting symptoms, were collected from fields across various locations in Adim, Cross River State. They were rinsed under tap water and dried between the folds of blotting paper. They were then placed in small plastic bags and stored at 4^{o} C until further processing.

Serological indexing for virus detection. The Antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA) method was used for the detection of CMV in collected leaf samples using a modified protocol described by Longe *et al.* (2022). From each of the leaf sample, 100 mg were ground in 1 ml of antigen extraction/coating buffer (1:10 w/v). One hundred microlitres of the antigen ground was dispensed into each well of the polystyrene plates covered and incubated overnight at 4 °C, before being washed three times with 1x PBS-Tween by flooding for 3 minutes each time. The plate was drained, tap-dried and blocked with 200 µl per well of 3% (w/v) dried non-fat skimmed milk in PBS-Tween. The plate was covered, incubated at 37°C for 30 minutes, emptied and tap dried. A total of 100 µl of polyclonal antibody was added to each well and diluted with conjugate buffer in the ratio 1:2000.

The plate was covered and incubated at 37°C for 1 hr, then washed three times with PBS-Tween by flooding for 3 minutes each time before tap drying. A total of 100 µl of goat anti-rabbit alkaline phosphatase conjugate diluted in conjugate buffer was added into the each well.

One hundred microlitres of p-nitrophenyl phosphate substrate (0.5 mg ml^{-1}) in substrate buffer was added per well. The plate was placed in the multiscan ELISA plate reader provided with 405 nm filter and the reading obtained after 1 hr and overnight. Samples with recording more than twice the reading of the healthy control were considered CMV positive.

Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR). Four seropositive samples with the highest ELISA readings were selected for molecular characterisation of the detected CMV. Total RNA was extracted from infected leaf tissues of *P. foetida* plants using cetyltrimethylammonium bromide protocol as described by Abarshi *et al*. (2010).

Total nucleic extracts from the leaf tissues were converted into complimentary DNA by the enzyme reverse transcriptase before amplification using a one-step RT-PCR procedure. The procedure was performed by using CMV forward primer F4 5'-GCCGTAAGCTGGATGGACAA-3' and reverse R4 5'-CCGCTTGTGCGTTTAATG GCT-3' (Apalowo *et al.*, 2022) resulting in ~ 500 bp amplicons. The total RT-PCR reaction mixture (50 µl) contained 1µl each of CMV primer forward and reverse, 5 x Go taq green buffer [(10.0 µl), $MgCl_2$ (3.0), dNTPs (1.0 µl), reverse transcriptase (0.24 µl), taq DNA polymerase (Promega) (0.24 µl), sterile

distilled water (30.52 µl) and nucleic acid extracts (3.0 µl)].

Amplification was carried out in a GeneAmp 9700 PCR system thermocycler (Applied Biosystem Incorporated, USA) using the following thermocyclic conditions: 42 ^{0}C for 30 minutes for reverse transcription, 94 ^{0}C for 3 minutes for initial denaturing, followed by 40 cycles of denaturing at 94 ^{0}C for 30 seconds, an annealing step at 40 ^{0}C for 30 seconds, an extension at 68 ^{0}C for 1 minute, and a final extension at 72 ^{0}C for 10 minutes ending the RT-PCR reaction.

The PCR reaction products were mixed with a loading dye, and separated on 1.5% agarose gel stained with ethidium bromide at 100 volts for 1 hour. The gel was subsequently visualised under UV light and photographed. A DNA molecular marker of 100 bp was used to estimate the size of the amplicons.

Sequencing and phylogenetic analyses. Two amplified fragments were selected and sent for sequencing. The obtained nucleotide and coat protein sequences having 501 bp and 497 bp respectively; were deposited to the GenBank database with ascension numbers MZ557308 and MZ557309 acquired for them.

Purified PCR amplicons were sequenced in both orientations using Sanger method at Inqaba Biotec West Africa Limited, Ibadan. The sequencing data were manually edited by using BioEdit software v.7.0.5 (Hall, 1999) The *P. foetida* isolate sequences were compared to known CMV sequences using basic local alignment search tool (BLASTn) program (www.blast.ncbi.nim.nih/Blast.cgi) available at the National Centre for Biotechnology Information (NCBI) and sequence homology was established. Multiple alignments of the nucleotide sequences were performed using the CLUSTAL-W program (Thompson *et al.,* 1994).

The phylogenetic relationship between the two *P. foetida* isolate with 29 selected CMV sequences belonging to subgroups IA, IB and II and obtained from GenBank, NCBI was determined. The evolutionary analysis was done using the software MEGA v. 7 (Kumar *et al*., 2013) using the maximum likelihood method while bootstrap values were calculated using 1000 random replications.

RESULTS

Detection by ELISA and PCR test. The ACP-ELISA results revealed that, the extracts strongly reacted positively to CMV specific antibodies, with the absorbance values ranging from between 1.62 and 2.071 optical density (O.D) values, which indicated the presence of CMV in *P. foetida*. Foliar symptoms associated with the plant were mild mosaic and leaf deformation (Fig. 1), and the obtained fragments from *P. foetida* isolate which corresponded to approximately 500 bp were successfully amplified using CMV-specific primer (Fig. 2). There was no amplification for samples tested for potyviruses, tymoviruses and carlaviruses.

Analysis of sequences. The two CMV isolates that were obtained, MZ557308 and MZ557309 had sequence lengths of 501 bp and 497 bp, respectively, with an encoded open reading frame (ORF) of a partial 1a protein containing 139 amino acid residues. The nucleotide and amino acid sequences of the isolates shared 96.78 - 96.81% and 94.81 - 95.69% similarities between them. The BLASTn analysis of the isolates from *P. foetida* revealed a nucleotide homology of 92.81 - 93.41% with a CMV isolate from Uganda (MG021460).

Pairwise sequence analyses of the two isolates revealed a sequence homology with isolates from Nigeria, Austria and Uganda at the nucleotide level (Fig. 3). The similarities of the sequences with CMV strains based on nucleotide comparison of partial RNA-1 genome ranged between 82 and 98%.

Table 1 and Figure 4 showed that the 31 isolates, including those retrieved from GenBank, were divided into four main phylogroups inconsistent with geographic origins and hosts, with each phylogroup sub-

Figure 1. Mild mosaic symptom of Cucumber mosaic virus on *P. foetida* from the field.

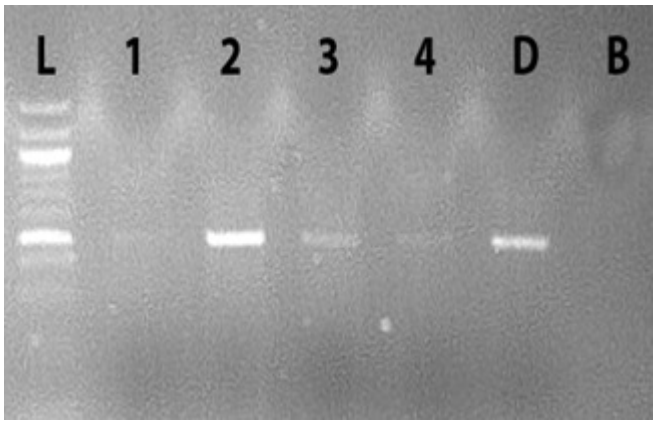

Figure 2. Detection of Cucumber mosaic virus by RT-PCR in *P. foetida*. The products were analyzed through electrophoresis on 1.5 % agarose gel. Wells L1-4 contain amplicons for CMV seropositive samples.

divided into several branches. Phylogroup IA included isolates from China, Ecuador, Australia, Taiwan, and USA. Phylogroup IB consists of isolates from South Korea, USA, Brazil, Hungary, France, Mexico, Japan, China and Spain. Phylogroups II consisted of isolates from United Kingdom, United States, Japan, Italy and Australia. The final phylogroup consist of isolates from Uganda, Austria and Nigeria. Unlike the pairwise comparisons, the phylogenetic tree reveals the isolate MZ557039 clustered closest with isolates HQ874434, MH798809 and MW655577 from Australia and Nigeria respectively. The other isolate MZ557038 was however closest to MZ557039.

DISCUSSION

Detection of CMV in *P. foetida*. Cucumber mosaic virus is notoriously known to infecting several crops, weeds and medicinal plants all over the world. In Nigeria, natural occurrence of CMV has been reported on a range of crops

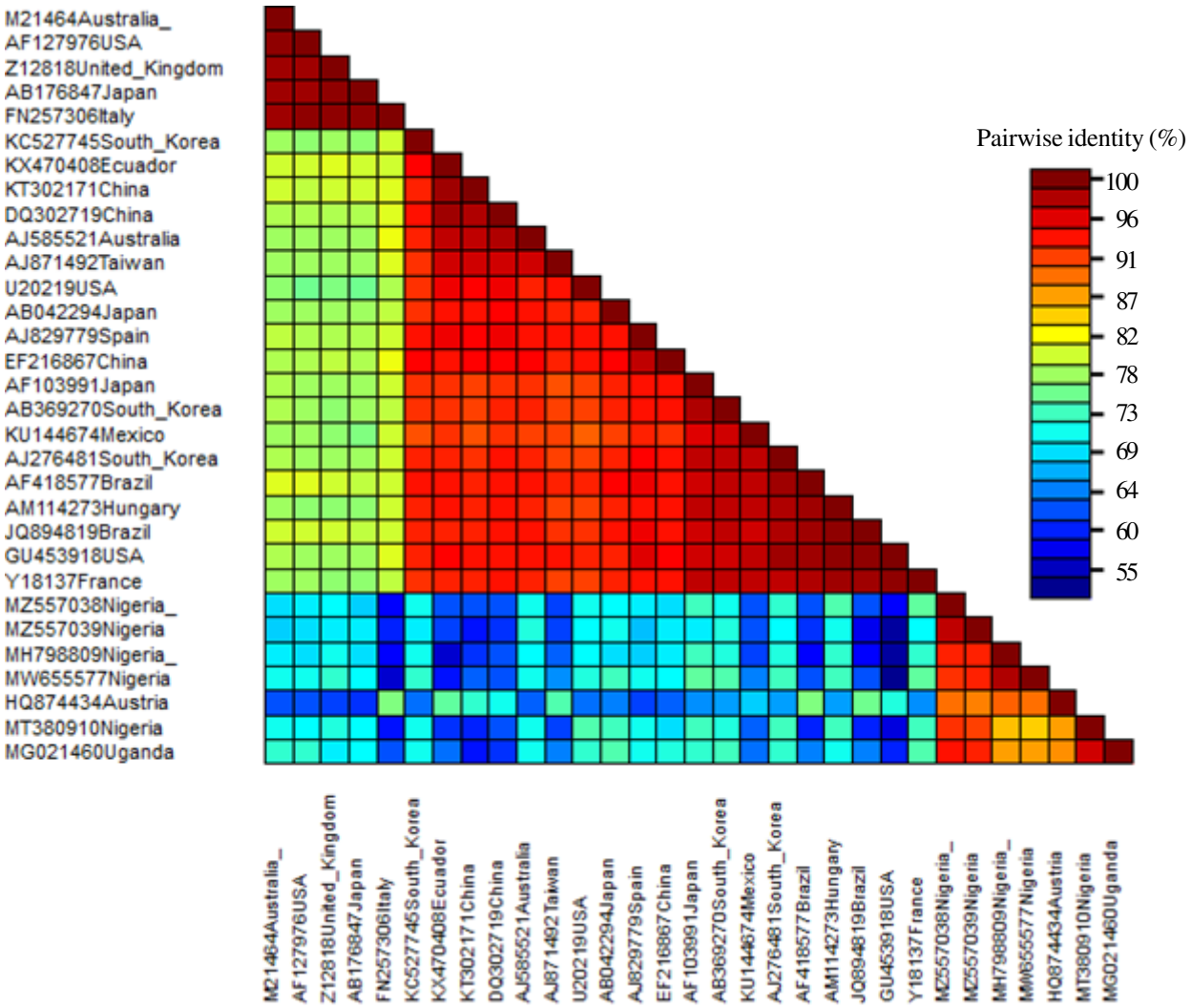

Figure 3. Pairwise sequence comparisons of MZ557038 and MZ557039 with representative sequences and CMV isolates obtained from NCBI GenBank. Coloured cells show pairwise identity score between two sequences.

such as *Solanum lycopersicon, Cucumis sativa, Ocimum gratissimum, Basella alba, Musa species, Talinium fruticosum, Telfairia occidentalis, Eleutheranthera ruderalis* and *Capsicum annuum* among others (Adediji, 2019; Arogundade *et al*., 2019; Eyong *et al*., 2020; Ekpiken *et al*., 2021; Apalowo *et al*., 2022; Longe *et al*., 2022). The present investigation revealed the natural occurrence of CMV on *P. foetida* with its detection and identification based on ELISA testing and sequencing of its partial genome.

In the present study, the isolates from the symptomatic leaf samples from *P. foetida* reacted positively with the CMV antiserum, but was negative for potyviruses. This is in agreement with results obtained by Arogundade *et al.* (2023) where no mixed infection of potyvirus was detected in *P. edulis*. Since only CMV was detected from the symptomatic leaves of the plant using ACP-ELISA and RT-PCR, the disease symptoms on the leaves can be associated with the presence of the virus and the plant confirmed as a new

TABLE 1. Nucleotide sequences of CMV coat protein in the GenBank used for comparison

Isolate name/strain	Host plant	Country	Subgroup	Genebank accession number
KW-EY-05	*Citrillus lanatus*	Nigeria	Un	MW655577
Gd	*Cucurbita pepo*	Austria	Un	HQ874434
Emma3	*Ocimum gratissimum*	Nigeria	Un	MT380910
-	-	Australia	II	M21464
Kin	-	United Kingdom	II	Z12818
243	Passion fruit	Australia	IA	AJ585521
Alla	*Allamanda cathartica*	Taiwan	IA	AJ871492
Fny	*Blephilia hirsuta*	USA	IB	GU453918
CRS-F2	*Passiflora foetida*	Nigeria	Un	MZ557039
CRS-F1	*Passiflora foetida*	Nigeria	Un	MZ557038
Pichilingue 1	*Passiflora edulis*	Ecuador	IA	KX470408
GF	*Solanum lycopersicum*	China	IA	DQ302719
PAE1	*Passiflora edulis*	Italy	II	FN257306
Dn4-4	*Musa paradisiaca*	China	IA	KT302171
26P_Ibi	*Capsicum annuum*	Nigeria	Un	MH798809
VAL90/1	*Solanum lycopersicum*	Spain	IB	AJ829779
-	Passion fruit	Brazil	IB	AF418577
1468	*Dianthus caryophyllus*	Mexico	IB	KU144674
RP16	*Capsicum annuum*	South Korea	IB	KC527745
Ug92	*Xanthosoma sp*	Uganda	Un	MG021460
LS	-	USA	II	AF127976
Mf	-	South Korea	IB	AJ276481
Pepo	*Cucurbita pepo*	Japan	IB	AF103991
CMV-Le02	*Solanum lycopersicum*	Hungary	IB	AM114273
V	*Nicotiana benthamiana*	South Korea	IB	AB369270
I17F	-	France	IB	Y18137
TN	*Solanum lycopersicum*	Japan	II	AB176847
Cb7	*Solanum lycopersicum*	China	IB	EF216867
CMV-Commelina-PR	*Commelina sp*	Brazil	IB	JQ894819
Ixora	*Solanum lycopersicum*	USA	IA	U20219
-	-	Japan	IB	AB042294

*Un- representing CMV isolates that did not align to known major subgroups

host of the virus. The stinking passionflower is mainly regarded as a medicinal plant, found in waste places and around the edges of farms as weeds, thereby constituting a potential reservoir for plant viruses including the cosmopolitan CMV.

Molecular properties of CMV in passionflower. The RT-PCR amplification and successful sequencing of the partial CMV RNA-1 fragment from *P. foetida,* confirmed the ACP-ELISA results and the constructed phylogenetic tree placed the virus isolates (MZ557038 and MZ557039) in a phylogroup consisting of mostly CMV isolates obtained from Nigeria, Uganda and Austria. These isolates did not align with any of the major CMV subgroups and are, therefore, described

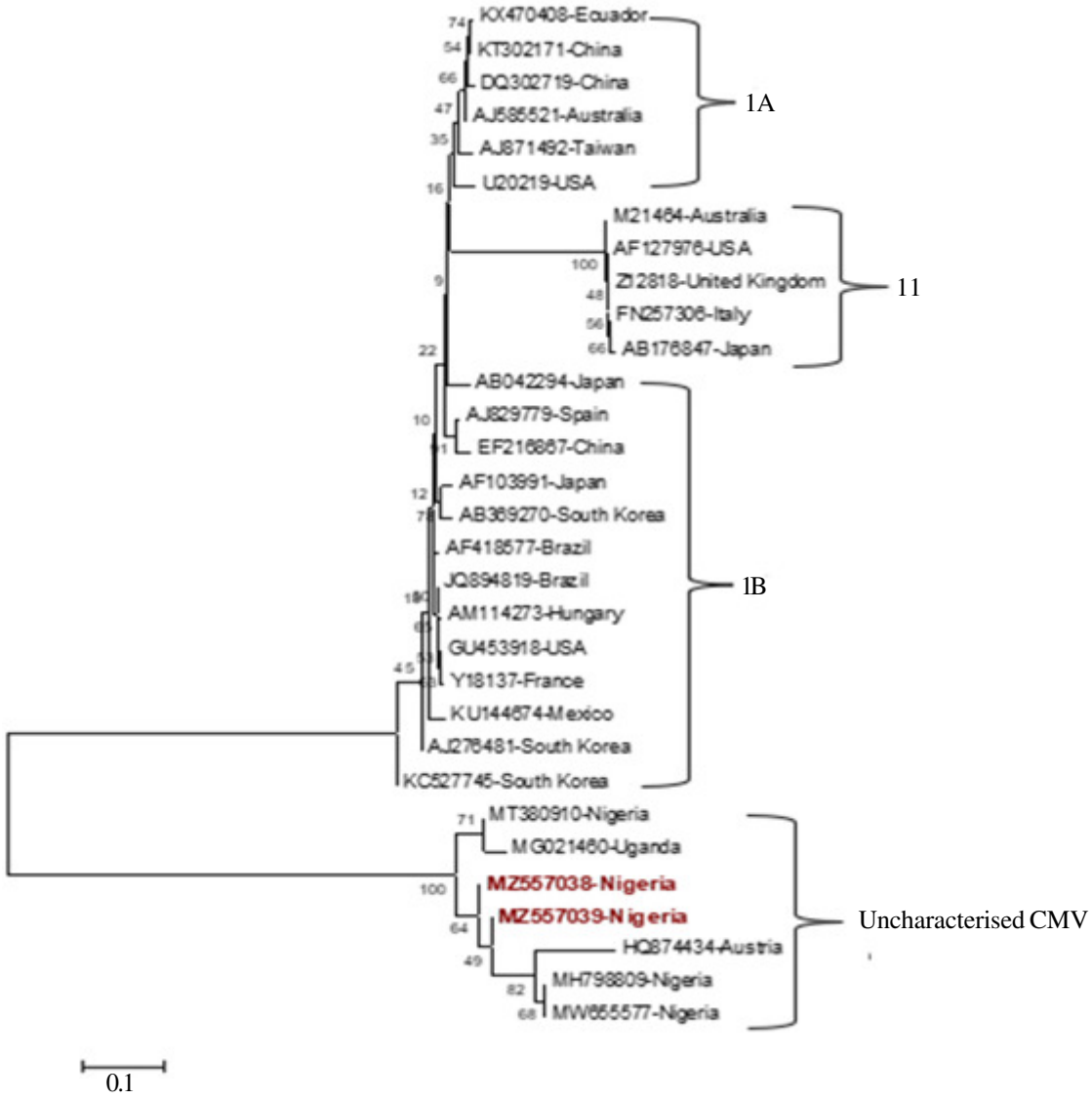

Figure 4. Phylogenetic tree of partial RNA-1 of *Cucumber mosaic virus* from *Passiflora foetida* in Nigeria.

in this study as uncharacterised CMV strains. However, they were clustered to the IB subgroup. Previously characterised CMV isolates, especially from Nigeria, have been placed in either subgroups IA, IB or II (Ayo-John and Hughes, 2014; Kayode *et al.,* 2014; Ekpiken *et al.,* 2021). The occurrence of subgroup IB in this study, and its prevalence in Nigeria, strongly supports an earlier report by Haase *et al*. (1989) which suggested that subgroup I isolates were more commonly found in the tropics and subtropics while subgroup II isolates were prevalent in the temperate regions.

Host and Strain categorisation of CMV. The results from this study showed that CMV isolated from *P. foetida* in Adim was more

closely related to those isolated from *Citrillus lanatus* (MW665577) and *Capsicum annuum* (MH798809) from Nigeria and *Cucurbita pepo* (HQ874434) from Austria. The virus is known to be ubiquitously found in several plants (Palukaitis *et al.,* 1992) and thus, the isolate similarities may not necessarily follow host delineations. The uncharacterised CMV isolates from *P. foetida* were clustered together with other isolates that did not exactly fit into any of the universally established subgroups but can be categorised as divergent members of subgroup IB due to their evolutionary relationship within the tree. A recent report by Apalowo *et al*. (2022) identified putative novel CMV strains from three hosts in Nigeria. The CMV isolates from this study clustered similarly with these strains and thus confirms the occurrence of divergent CMV isolates. Liu *et al.* (2009) described a putative subgroup III for CMV strains, while Wang *et al.* (2009) postulated a novel subgroup IC. Since the isolates in this study clustered away from the major subgroups, they may be considered as members of these putative subgroup III or IC.

The phenomenon of isolates of CMV deviating from established subgroups may not be unconnected to recombination of genes. Moyle *et al*. (2018) stated that recombination occurs in a CMV genome because of the possibility of RNA segments within a single genome clustering into dissimilar subgroups. This is especially present within the partial RNA-1 segment (Apalowo *et al.,* 2022), similar to the region targeted and amplified in this study. The phylogenetic differentiation of these isolates into a distinct uncharacterised subgroup putatively suggests the emergence of recombinants that differ from the conventional strain categorisation for CMV. Although explicit recombination analyses were not done in this study, its role in CMV diversity is well documented (Nouri *et al.*, 2014; Ouedraogo *et al*., 2019) and may be responsible for the atypical properties seen within the CMV isolates in this study.

CONCLUSION

This study revealed for the first time, the detection and characterisation of CMV in *P. foetida* leaves that showed mild mosaic symptoms using both ELISA and molecular tools. The presence of these symptoms has also been associated with CMV infection in passionflower. Molecular categorisation of the CMV isolates confirmed that they belong to subgroup IB, establishing the strain as the prevalent across various host plants in Nigeria. The knowledge of host range diversity and strain identification is important for robust detection and overall management of CMV infections to limit the debilitating impact of the virus. It is important that more research be carried out on CMV infecting *P. foetida* so that information on its distribution, transmission studies, and genomic diversity can be further established.

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